MODE OF ACTION OF N-METHYL-N'-NITRO-N-NITROSOGUANIDINE AS A MUTAGEN FOR SALMONELLA TYPHIMURIUM

by

SIU WAI SAE

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Approved by:

A. Eisenstark
Major Professor

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INTRODUCTION

For bacterial genetic studies it is helpful if mutant strains can be obtained efficiently. N-methyl-N'-nitro-N-nitrosoguanidine (NG)[1] appears to be one of the most powerful mutagens now known and is being used extensively for the induction of Salmonella typhimurium mutants (Eisenstark, Eisenstark and Van Sickle, 1965). In view of this, some quantitative information on the efficiency of the production of auxotrophic mutants is necessary. Therefore, studies were undertaken to find the growth phase of cells, the medium, pH, concentration of NG and time of treatment with NG which would give an optimal yield of auxotrophic mutants.

Although it is being used as a mutagen, studies on the mode of action of NG have not been reported. A review of its chemical properties shows that NG decomposes easily, and one of the decomposition products, diazomethane, is a good alkylating agent which can alkylate nucleic acids (Kriek and Emmelot, 1963, 1964); but no interaction of NG with DNA has been reported. In this thesis, besides the optimal condition studies, attempts to reveal the mutagenic mode of action of NG through the studies of its effects on bacterial cells, bacteriophage (in vivo and in vitro) and DNA (in vitro) are described and discussed.

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[1]Following is a list of all symbols and abbreviations in alphabetical order used throughout this thesis: arg (arginine); DNA (deoxyribonucleic acid); ENG (N-ethyl-N'-nitro-N-nitrosoguanidine); gua (guanine); met (methionine); NG (N-methyl-N'-nitro-N-nitrosoguanidine); RNA (ribonucleic acid); thy (thymine); try (tryptophan).

REVIEW OF LITERATURE

The compound N-methyl-N'-nitro-N-nitrosoguanidine (NG) was first prepared by McKay and Wright (1947), and has been used for the preparation of diazomethane by distillation.

In the course of investigation of the action of a selected group of anticancer agents on genetically stable mutants of Escherichia coli, Mandell, Woody and Greenberg (1961) noted that resistant mutants had been induced by one of them, NG. The hypothesis that NG was a general mutagen was confirmed by demonstrating the presence of auxotrophs among the survivors of the NG treatment (Mandell and Greenberg, 1960). By further treatment of these resistant mutants (first-step mutants, S/Ng1), the second-step mutants (S/Ng2) were obtained. These S/Ng2 mutants were more resistant to NG than S/Ng1 mutants. Since then, published works involving the mutagenic effects of NG on bacteria have been few (Eisenstark and Van Sickle, 1963; Jacob, Brenner and Cuzin, 1963; Nishioka and Eisenstark, 1964; Adelberg, Mandel and Chen, 1965; Eisenstark et al., 1965; Eisenstark, 1965).

Adelberg and his co-workers (1965), in their investigation on optimal conditions for mutagenesis by NG in E. coli K12, stated that NG might react with DNA (replication not necessary) in such a way as to promote template errors for subsequent replication.

Eisenstark et al. (1965) studied a number of multisite mutants induced by NG and diethylsulfate and found that these two chemical mutagens had a similar mode of action in that the mutants could be reverted by both mutagens.

There are some studies on the general mechanism of action of NG mainly as a radiomimetic compound (a compound that produces biological effects similar to those produced by radiation). The first-step mutants (S/Ng1) and

second-step mutants (S/Ng2) of E. coli strain S resistant to NG (Mandell et al., 1961) were tested for cross-resistance to other diazoalkane-yielding compounds, N-1-alkyl-substituted nitrosoguanidine, other radiomimetic compounds such as nitrogen mustard, and ultraviolet radiation. It was found that S/Ng2 was more sensitive to non-structurally similar radiomimetic compounds than S/Ng1. From these results the authors suggested that there could be at least two types of events in the course of developing increased resistance to NG. The first step might involve some physiological change which makes the bacteria to some degree resistant to a variety of chemicals. The second step might involve a stereo-specific mechanism on NG per se or structurally related chemicals; therefore, non-structurally similar radiomimetic compounds such as nitrogen mustard would be more inhibitory to S/Ng2 than to S/Ng1.

Evidence that NG acts like a mustard type alkylating agent is that NG inhibition against E. coli is reversed nonspecifically by a variety of amino acids and by sulfhydryl agents (Skinner et al., 1960; Greenberg and Morris, 1961). The interaction of NG with -SH and $-NH_2$, two key groups in biological systems, may well be responsible for its cytotoxic effects.

Gichner, Michaelis and Rieger (1963) used NG to induce chromosomal aberrations in Vicia faba and suggested that NG has a mode of action similar to N-methylphenylnitrosamine and cupferron, while its mutagenic activity on Arabidopsis showed that its extremely low relative toxicity is similar to ethyl methanesulfonate and to nitrosomethylurea, but clearly differs from nitrogen mustard (Müller and Gichner, 1964).

One other property of NG that has been studied is its anticancer effect; it has been found to have anti-ascites tumor action in mice (Greene and Greenberg, 1960) and to have antileukemic activity (Skinner et al., 1960; Hyde et

al., 1962). No interaction of NG with DNA or its bases has been reported.

In order to compare NG with alkylating agents, some of the properties of these compounds will be reviewed here.

The general biological effects of alkylating agents (Ross, 1962; Wheeler, 1962) are: (1) delay or prevention of mitosis in "resting" cells, (2) mutagenic effects, and (3) cytotoxic effects. These effects occur in that order as the dosage is increased.

The cytotoxic effects of alkylating agents on higher organisms have been considered to be caused by chromosome breakage (Auerbach, 1958) and random interaction with sulfhydryl groups (Craddock, 1964; Mizrahi and Emmelot, 1962; Mizrahi and Emmelot, 1963), proteins (Magee and Hultin, 1962), nucleic acids (Magee and Farber, 1962), and perhaps also phospholipids (Mizrahi and Emmelot, 1962).

Reiner and Zamenhof (1957), by treating transforming DNA with dimethylsulfate, obtained 7-methylguanine and 7-methyladenine from the products of hydrolysis. Purines were found to be much more reactive than pyrimidines. Loveless first studied the mutation and inactivation of T2 bacteriophage in vitro by the alkylating agents ethyl methanesulfonate (Loveless, 1958) and di(2-chloroethyl)methylamine (Loveless and stock, 1959; Loveless, 1959). They concluded that the enhancement of host-range mutants and inactivation of phage were due to ethylation of phage DNA. Bautz and Freese (1960) determined the specificity of ethyl methanesulfonate and showed that the main mutagenic effect of ethylation was probably due to the elimination of 7-ethylguanine from DNA, giving rise to both transition and transversion. Ethylation of the primary phosphate group of DNA, which breaks the phosphate-sugar backbone in a way similar to the decay of incorporated P^{32}, has not been found to

be mutagenic. Lawley and Brookes (1963) treated nucleic acids with S^{35}-mustard gas, C^{14}-methyl methanesulfonate and C^{14}-ethyl methanesulfonate; in all these cases, alkylation resulted in destabilization of the nucleosides or the corresponding moieties in the nucleic acid. Their studies in the inactivation of bacteriophages T2 and T_4 (Brookes and Lawley, 1963) showed that the sites of DNA alkylated within the phage and their relative reactivities were identical with those of isolated DNA. The results suggested that inactivation by difunctional agents results from inter-strand crosslinking of DNA. With monofunctional agents it seemed likely that the principal mode of inactivation resulted from degradation of the DNA subsequent to its alkylation. The fact that the difunctional agents generally exert a more powerful cytotoxic action than the monofunctional may be related to the ability of difunctional agents to cross-link the twin strands of DNA (Brookes and Lawley, 1961). Brookes and Lawley considered that depurination after alkylation was a process that potentiated the inactivation of phage rather than inducing mutation, and favored the hypothesis that there is retention of alkylated purine bases which causes incorrect base-pairing between ionized 7-alkylguanines and thymine (Brookes and Lawley, 1964).

The methylation of RNA (Kriek and Emmelot, 1963) and the methylation of DNA (Kriek and Emmelot, 1964) with diazomethane gave 1-methylguanine, 7-methylguanine, 1,7-dimethylguanine, 7-methyladenine, and 3-methyladenine from the products of hydrolysis. It has been reported that the strong mutagenic and carcinogenic effects of N-nitroso- compounds is due to the formation of diazoalkane which alkylates nucleophilic centers of cells (Druckrey et al., 1963; Kriek and Emmelot, 1963). With dimethylnitrosamine, enzymatic oxidative N-demethylation to release an agent which methylates amino acids and nucleic

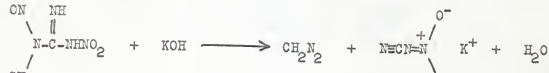
acids in vitro and in vivo has been postulated (Magee and Hultin, 1962; Magee and Farber, 1962). Loprieno et al. (1964), in their studies of lethal and mutagenic effects of N-nitroso-N-methylurethane and N-nitroso-N-ethylurethane in Collectotrichum cocodes, also suggested that the alkylating agent in the cell might be diazomethane formed under alkaline conditions.

NG itself is an alkylating agent as is shown by the following reaction:

R' = an alkyl, aralkyl, or aryl group.

NG reacts with amines to yield N-substituted N'-nitroguanidines with the alkylation of a second molecule of the amine (McKay, 1952). Skinner et al. (1960) found similar reactions occurring if they used amino acids instead of amines.

NG decomposes under alkaline conditions to yield diazomethane (McKay, 1952):



The diazomethane (CH_2N_2) formed is an active methylating agent.

Moreover, the desnitroso and N-amino analogues of NG which had lost their alkylating ability and the ability to form diazomethane were found to be inactive on E. coli (Greenberg and Morris, 1961). The alkylating properties of NG thus lead one to postulate that the mode of action of NG in inducing

lethality and mutations in the biological system is similar to that of the other alkylating agents. However, direct experimental evidence similar to that obtained for the other alkylating agents reviewed above has yet to be obtained.

MATERIALS AND METHODS

Bacteria

The wild type prototroph used in all the optimal condition studies was Salmonella typhimurium LT2. The mutant strains used were: KSU#244 (gua-2), for reversion of marker by NG; and KSU#2296 (try-2, met-22, arg, thy), for glucose starvation, thymine starvation, amino acid starvation and mutation studies with NG. The hosts for the bacteriophages T4D and ØX174 were Escherichia coli S/6/5 and E. coli C strain, respectively; both were provided by Dr. W. Bode. Phage ØX174 resistant strains were derived from the E. coli C strain.

Bacteriophage

Temperate phage P22 stock was prepared on S. typhimurium LT2. Phage T4D (a lysozyme and temperature sensitive mutant), and ØX174 (wild type) were obtained from Dr. W. Bode.

Media

Nutrient broth. Nutrient broth was prepared by dissolving 8 g dehydrated Bacto-nutrient broth and 5 g sodium chloride in 1 liter of deionized distilled water.

Hershey's nutrient broth. Hershey's nutrient broth was prepared by dissolving 8 g dehydrated Bacto-nutrient broth, 5 g Bacto-peptone, and 5 g sodium chloride in 1 liter of deionized distilled water. After autoclaving, 5 ml of a 20% (w/v) glucose solution was added.

Nutrient agar. Nutrient agar was prepared by dissolving 15 g Bacto-

agar into 1 liter of nutrient broth.

Nutrient overlay. Nutrient overlay was prepared by dissolving 8 g Bacto-agar into 1 liter of nutrient broth.

Tryptone agars. Tryptone agar for T4D phage assay was prepared by dissolving 13 g Bacto-tryptone, 6 g sodium chloride, 1 g glucose and 12 g Bacto-agar in 1 liter deionized distilled water.

Tryptone agar for ØX174 phage assay was prepared by dissolving 10 g Bacto-tryptone, 5 g Bacto-yeast extract and 12 g Bacto-agar in 1 liter of deionized distilled water; after autoclaving, 25 ml of a 50% (w/v) glucose solution and 2.5 ml 1 M $CaCl_2$ solution were added.

Minimal medium. Minimal medium was made double strength by dissolving 21 g K_2HPO_4, 9 g KH_2PO_4, 2 g $(NH_4)_2SO_4$ and 0.94 g sodium citrate in 1 liter of deionized distilled water. It was diluted 1:1 with sterile deionized distilled water before use. Glucose was then supplemented by adding 10 ml 40% (w/v) glucose solution before use. Other supplements, unless specified, were added to a final concentration of 20 µg/ml of medium.

Minimal agar. Minimal agar was prepared by dissolving 35 g Bacto-agar in 1 liter of deionized distilled water. It was diluted 1:1 with the double strength minimal medium before use. Supplements were added in the same quantities as for the supplemented minimal medium.

Double-enriched agar. Double-enriched agar was prepared by adding 20 ml nutrient broth and 8 ml of 40% (w/v) glucose solution to 800 ml of minimal agar before pouring into plates. Other supplements were added in the same quantities as for supplemented minimal medium.

Physiological saline. Physiological saline was prepared by dissolving 8.5 g sodium chloride into 1 liter of deionized distilled water.

Tris-maleic buffer. Tris-maleic buffer (TM buffer) was prepared by dissolving 6.05 g 2-amino-2(hydroxymethyl)-1,3-propanediol, 5.8 g maleic acid, 1.0 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, 7.2 mg $Ca(NO_3)_2 \cdot 4\ H_2O$ and 0.25 mg $FeSO_4 \cdot 7H_2O$ in 1 liter of deionized distilled water; the solution was then adjusted to pH 7.2 with NaOH solution. This medium was diluted 1:1 with deionized distilled water before use.

All media were sterilized by autoclaving at 110°C and 15 lb pressure for 15 minutes.

Chemicals

N-methyl-N'-nitro-N-nitrosoguanidine was purchased from the Aldrich Chemical Co., Milwaukee, Wis. It was recrystallized from methanol. Fresh solutions in sterile deionized distilled water or in sterile minimal medium (pH 5.5 or pH 7.0) were made for each use and were used without sterilization.

N-ethyl-N'-nitro-N-nitrosoguanidine was also obtained from the Aldrich Chemical Co., and was prepared, without recrystallization, by dissolving in sterile, deionized distilled water.

Highly polymerized salmon sperm DNA and synthetic guanine were purchased from Mann Research Lab., Inc., New York, N. Y. Adenine was obtained from California Corporation for Biochemical Research, Los Angeles, California.

Method for Shifting Cells to New Medium

A cell suspension containing 1 to 2 x 10^8 cells/ml (not more than 200 ml in each batch) was poured onto a sterile, wet S&S Bact-T-Flex membrane fil-

ter (type B-6, 150 mm, 0.45 μ), sitting on a brass filter funnel. Suction was applied to the filter by means of a water aspirator, and the cells were washed by pouring over them some of the warmed (37°C) medium into which they were to be transferred. The cells adhered to a small area near the center of the filter due to the suction applied. After washing, the filter was trimmed to remove the portion to which no cells adhered; this trimmed filter with cells was placed in a warm (37°C) resuspending medium, and shaken on a Vortex mixer. The resulting cell suspension was transferred to a warm (37°C) container. This technique was also employed in washing NG from the cells, using Gelman metricel filters, type GA-7, 0.30 μ.

Treatment of Cells with NG in Liquid Media

A refrigerated, saturated bacterial culture was diluted with fresh nutrient broth to about 1 x 10^7 cells/ml. It was incubated at 37°C with bubbling aeration until the cells numbered 1 to 2 x 10^8/ml. A 20 ml sample of culture was centrifuged at 6,000 x G for 5 minutes at room temperature. The cells were washed by resuspending in the medium in which they were to be treated with NG, centrifuged again and resuspended in the same medium to the original volume (20 ml). A 0.05 to 0.1 ml aliquot of freshly prepared NG solution was added to 4.0 ml of cell suspension to give the required NG concentration. After incubating at 37°C with air bubbling, dilutions into saline were made without washing. In plating for revertants of strain KSU#244 (gua-2), no dilution was made before plating on minimal agar.

Determination of Survival and Mutagenesis

The number of survivors after a NG treatment was determined by plating the treated cells on nutrient agar; the colonies which appeared on the nutrient

agar were scored as survivors.

The number of induced revertants of strain KSU#244 (gua-2) was determined by plating NG treated cells on minimal agar supplemented with glucose; colonies appearing on the minimal agar were scored as revertants. Cells not treated with NG gave a background frequency of spontaneous gua-2 reversion of less than 2×10^{-7} in all experiments.

To determine auxotrophy, a sample of each colony from the survival plate (containing not less than 100 colonies) was inoculated onto nutrient agar and minimal agar plates, using sterile toothpicks. Isolates showing growth on nutrient agar plates but not on minimal agar plates were scored as auxotrophs.

Treatment of Bacteriophage with NG in vitro

To the phage lysate, NG solution was added at a final concentration of 200 to 400 µg/ml. After incubating at room temperature for 20 to 90 minutes, the culture was diluted 1:10 into ice-cold 0.1 M Tris-buffer, pH 8.5, and plated on tryptone agar within 15 minutes, using the soft agar overlay method with the appropriate hosts.

Treatment of Bacteriophage with Nitrous Acid in vitro

The reaction mixture consisted of, by volume, 70 parts 0.2 M sodium acetate buffer at pH 4.0, 10 parts 0.5 M $NaNO_2$, and 20 parts phage lysate. The phage was treated at room temperature (26°C) for 20 minutes. The mutagenic treatments were terminated by dilution into ice-cold 0.1 M Tris-buffer, pH 8.5; the phage were then plated on tryptone agar, using the soft agar overlay method with the appropriate hosts. This method was developed by Tessman, Poddar and Kumar (1964).

Treatment of Bacteriophage with NG in vivo

A refrigerated culture of E. coli C was diluted to about 1 x 10^7 cells/ml in fresh Hershey's nutrient broth, and was grown to 1 x 10^8 cells/ml. NG was added at a final concentration of 400 µg/ml. After 5 minutes the culture was infected with ØX174 at a multiplicity of infection of 1. After 15 minutes the infected culture was washed and resuspended in warm (37°C) Hershey's nutrient broth. Incubation with vigorous aeration was continued until the culture became clear (about 1 hour). The phage lysate was centrifuged to get rid of bacterial debris, and chloroform was added to prevent bacterial growth. Assays for plaque forming units on the wild type host and host-range mutants on the mutant hosts were performed immediately on tryptone agar plates.

Method for Selecting Phage Mutants

Reversion of the temperature sensitive mutant of T4D was observed by plating the treated phage onto two sets of tryptone agar plates with host E. coli S/6/5. Both sets of plates were incubated at 30°C for 1 hour; then, one set was transferred to 45°C until plaques appeared. Control plates showed a spontaneous reversion rate of about 5 x 10^{-5}.

The resistant host strains for ØX174 were obtained by isolating colonies from an E. coli C strain culture confluently lysed on a tryptone agar plate after a high multiplicity of infection by wild type ØX174 phage. Phage that grew on these resistant strains of E. coli were scored as host-range mutants of ØX174.

Treatment of DNA with NG

Highly polymerized salmon sperm DNA (5.0 mg) was dissolved in 5 ml of 0.01 M sodium phosphate buffer, pH 6.8. To this solution was added 5.0 ml of

NG solution (0.0147 g, 100 μmoles final concentration in phosphate buffer). The mixture was incubated with shaking at 37°C for 30 minutes. The DNA was then precipitated with 20 ml of cold 95% ethanol, and was hydrolyzed in 1 N HCl solution (5 ml) for 20 minutes at 100°C.

Column Chromatography

A cation exchange column (16 cm x 1 cm) of Dowex 50W X 8 (200 to 300 mesh) in the H^+ form was washed with about 1 liter of 0.5 N HCl solution. The DNA hydrolysate was applied to the column and was eluted at room temperature, 25 to 26°C, with 2 N HCl solution. Fractions of 5 ml were collected at 5 minute intervals. The fractions were measured for absorbance at 260 mμ with a Beckman DK-2A spectrophotometer (Fig. 5).

Paper Chromatography

The fractions collected from column chromatography of the DNA lysate which absorbed at 260 mμ were pooled together and evaporated under reduced pressure to about 1 ml. These concentrated fractions were then applied to Whatman No. 1 filter paper for descending chromatographic separation. Authentic guanine and adenine samples were run on the same paper as controls. The solvent system consisted of a mixture of 680 ml isopropanol, 164 ml concentrated HCl (sp. gr. 1.19) and enough water to bring the total volume to 1 liter (Wyatt, 1951).

The spots were located under ultraviolet irradiation (254 mμ), cut out, and eluted with a 0.1 N HCl solution. The ultraviolet absorption spectra of the material eluted from these spots were obtained with a Beckman DK-2A spectrophotometer. Spectra of authentic samples of bases in 0.1 N HCl solution were also obtained as controls.

Infra-red Absorption Spectroscopy[2]

The fractions of the NG treated DNA hydrolysate eluted from the paper chromatogram were evaporated to dryness under reduced pressure, washed once with distilled water and evaporated to dryness again, and were then dried in an evaculate desiccator. KBr pellets of the dried guanine and adenine fractions thus obtained were prepared and spectra were obtained with a Perkin-Elmer 137 sodium chloride spectrophotometer.

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[2]Thanks are due to Mr. S. Nukina, Department of Chemistry, Kansas State University, for taking the infra-red absorption spectra.

RESULTS

Lethal and Mutagenic Effects of NG on Salmonella typhimurium

Effect of NG on a S. typhimurium auxotrophic mutant in the absence of glucose in the treatment medium. To study the effect of the lack of an external energy source on the bacterial cells during the NG treatment, a logarithmically growing culture of S. typhimurium KSU$^{11}_{11}$2296 carrying met-22, try-2, arg, and thy markers was shifted from the growth medium to a minimal medium lacking glucose and supplemented with methionine, tryptophan, arginine and thymine; and NG was added to a final concentration of 20 µg/ml. Samples were withdrawn and plated on nutrient agar for survival counts, and on supplemented, double-enriched minimal agar to obtain auxotrophic mutants. After 80 minutes, glucose was added to the starving cultures.

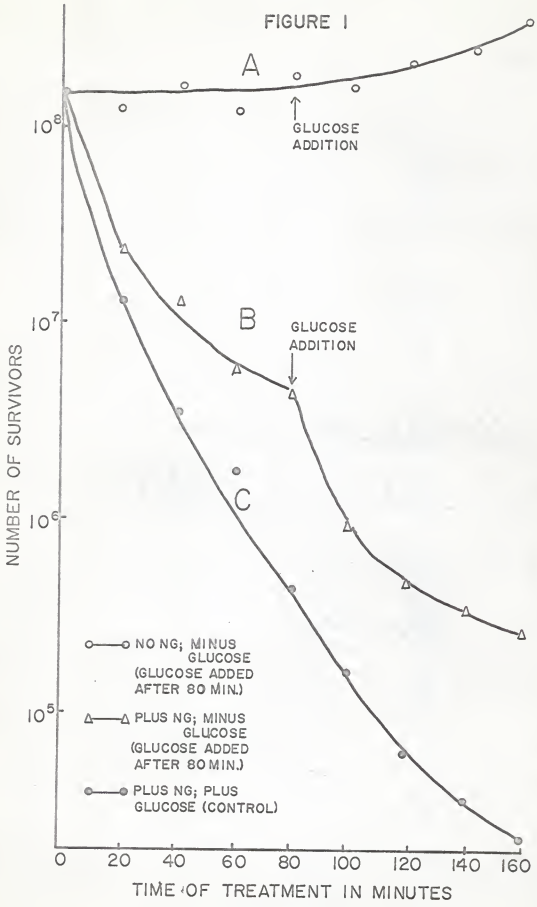
The lethal effects of NG are shown in Fig. 1. Curve B shows the number of survivors in the glucose-lacking medium decreasing at a slower rate than that of the control (Curve C), in which both glucose and NG were present. After the addition of glucose at 80 minutes, the number of survivors decreased at a considerably higher rate than when glucose was absent. This indicates that NG is more effective in killing metabolizing cells than resting cells. This may be due to a more active uptake of NG after glucose was added to the medium.

About 7 to 11 per cent auxotrophic mutants were obtained among the survivors after 20, 60, 100, and 160 minutes of treatment with 20 µg/ml of NG in the absence as well as in the presence of glucose in the medium. Therefore, the induction of mutations did not require cells to grow and metabolize in the presence of the mutagen.

Figure 1

Lethal effect of NG on S. typhimurium KSU#2296 (try-2, met-22, arg, thy) in the presence and absence of glucose. Cells were treated in a minimal medium with 20 µg/ml of NG; glucose was added to the medium after 80 minutes, except in the control which contained glucose throughout the experiment.

FIGURE 1


A
GLUCOSE ADDITION
B
GLUCOSE ADDITION
C
NUMBER OF SURVIVORS
10^8
10^7
10^6
10^5
NO NG; MINUS GLUCOSE (GLUCOSE ADDED AFTER 80 MIN.)
PLUS NG; MINUS GLUCOSE (GLUCOSE ADDED AFTER 80 MIN.)
PLUS NG; PLUS GLUCOSE (CONTROL)
0 20 40 60 80 100 120 140 160
TIME OF TREATMENT IN MINUTES

Effect of NG on a S. typhimurium amino acid requiring mutant in the absence of amino acids in the treatment medium. To determine whether NG can induce lethality and mutation in the absence of protein synthesis, S. typhimurium KSU#2296 (met-22, try-2, arg and thy) was treated with 20 µg/ml of NG in a minimal medium (supplemented with glucose and thymine) without amino acids. This strain is known to be stringent in protein synthesis[3]; that is, protein synthesis will stop almost completely in the absence of the required amino acids in the growth medium. However, the cells remain viable and will start synthesizing protein and multiply when amino acids are added (Fig. 2, Curve A). Cells were taken out at various intervals and plated on nutrient agar for viable cell counts and on supplemented, double-enriched agar for auxotroph counts.

Fig. 2, Curve B shows that in the absence of amino acids the cells were killed at a slower rate as compared to the control (Curve C) in which amino acids were present. The addition of amino acids after 85 minutes induced a considerable decrease in the survival rate indicated by the latter part of Curve B.

NG is also able to induce mutation (7 to 11 per cent auxotrophs among survivors) in this amino acid starved culture. The addition of the amino acids did not give a significant increase in per cent auxotrophs among the survivors as compared to that produced in the absence of amino acids in the treatment medium.

Effect of NG on a S. typhimurium thymineless mutant in the absence of

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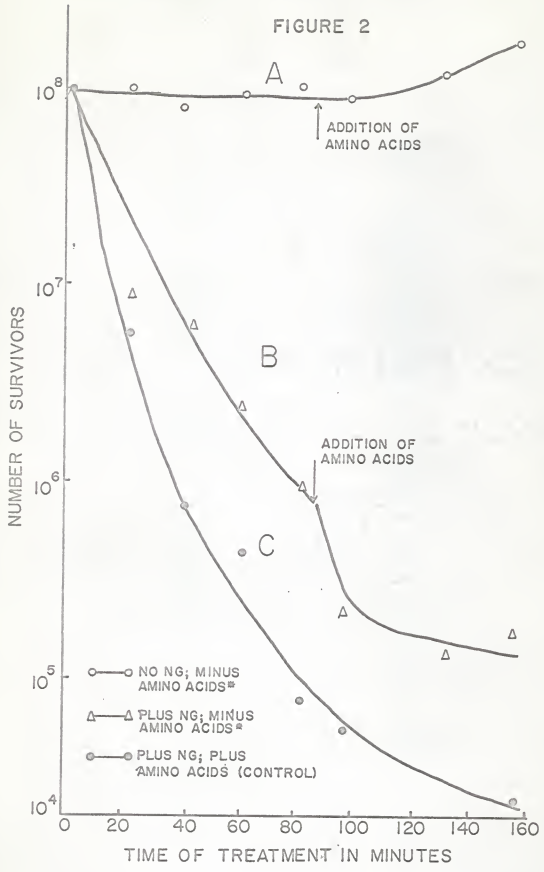
[3]Tested by Mr. Hilary Chan, Department of Physics, Kansas State University.

Figure 2

Lethal effect of NG on S. typhimurium KSU#2296 (try-2, met-22, arg, thy) in the presence and absence of the amino acids (tryptophan, methionine, and arginine). Cells were treated in a minimal medium with 20 µg/ml of NG; the amino acids were added after 85 minutes; the control contained the amino acids throughout the experiment.

FIGURE 2



*AFTER 85 MINUTES AMINO ACIDS WERE ADDED.

thymine in the treatment medium. To show that NG is capable of inducing lethality and mutation in cells which are not synthesizing DNA, S. typhimurium KSU#2296 (met-22, try-2, arg and thy) was used. Since the strain is stringent in DNA synthesis[4], DNA synthesis is stopped completely after shifting to a thymineless minimal medium. NG was then added. Samples were taken at 20 minute intervals and plated on nutrient agar for survivors and on supplemented, double-enriched minimal agar for induced auxotrophs which appeared as small colonies.

Fig. 3a shows the lethal effect. Without DNA synthesis the number of survivors at various times (Curve B) was significantly higher than the number of survivors in the control (Curve C) in which DNA synthesis was occurring. Thymineless death (Curve A) may have contributed to the steepness of the slope of the latter part of Curve B as compared to that of Curve C.

The mutagenic effect of NG on this thymineless mutant is shown in Fig. 3b. The number of small colonies (auxotrophic clones) which appeared on the double-enriched agar plates supplemented with methionine, tryptophan, arginine, thymine and glucose was divided by the total number of colonies on the plate to give the fraction of small colonies among the survivors. This fraction was plotted against time of treatment with NG. After 80 minutes of thymine starvation in the presence of NG, thymine was added without removing the NG. As is seen in Fig. 3b, there was an increase in the fraction of small colonies among the survivors. This indicates that the addition of thymine, and hence the resumption of DNA synthesis, rendered NG more effective in inducing mutation. The control with no NG added yielded no small colonies in more than 1000 colonies examined.

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[4]See footnote 3.

Figure 3a

Lethal effect of NG on S. typhimurium KSU#2296 (try-2, met-22, arg, thy) in a minimal medium lacking thymine. Cells were treated with 20 µg/ml of NG.

FIGURE 3a

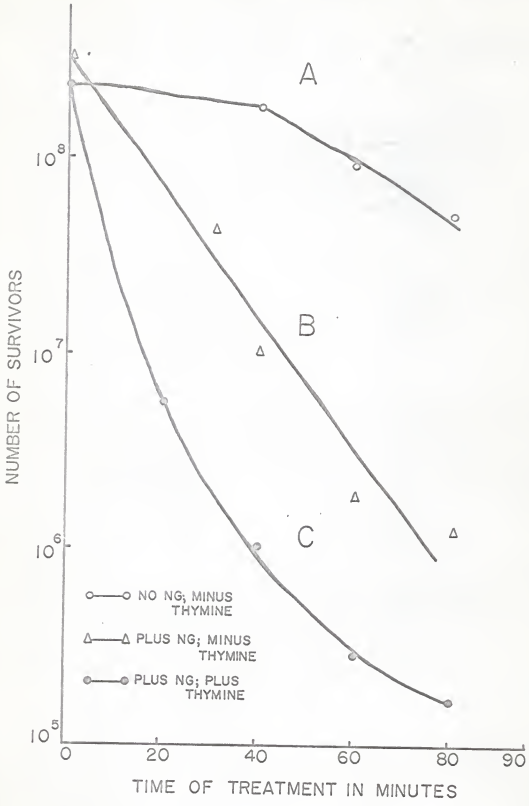

A
B
C
10^8
10^7
10^6
10^5
NUMBER OF SURVIVORS
NO NG; MINUS THYMINE
PLUS NG; MINUS THYMINE
PLUS NG; PLUS THYMINE
0
20
40
60
80
90
TIME OF TREATMENT IN MINUTES

FIGURE 3b

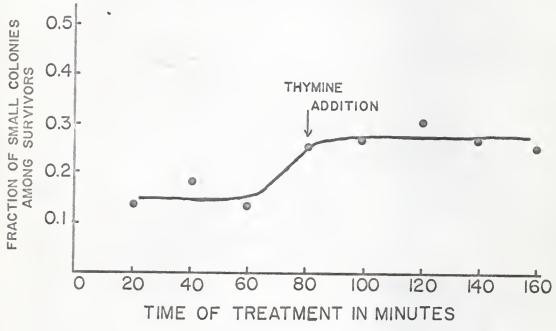


Figure 3b

Mutagenic effect of NG on S. typhimurium KSU#2296 (try-2, met-22, arg, thy) in the presence and absence of thymine. Cells were treated with 20 μg/ml of NG in a minimal medium; thymine was added after 80 minutes.

Effect of phases of growth on survival and reversion in the presence of NG. This experiment was designed to observe the effectiveness of NG on induction of reversions at different phases of growth. The gua-2 mutant of S. typhimurium was used because it has a moderate reversion rate in the presence of NG, but a low spontaneous reversion rate.

Fig. 4 shows an increase from 1 to 10 per cent in survival as the cells go from the lag phase to the logarithmic phase, while the reversion rate decreased after entering into the logarithmic phase. This indicates that during the late-lag and early-log periods of growth, in which metabolic rate per cell is greatly increased (Clifton, 1957), NG is most effective both in killing the cells and in inducing mutation. A gradual increase in survival percentage and a gradual decrease in reversion rate during the logarithmic phase and the stationary phase may be accounted for by a decrease in metabolic rate per cell. The decrease of metabolic rate per cell during the logarithmic phase is a common observation (Clifton, 1957).

As a result of this experiment, it was decided that for all subsequent experiments, a logarithmic culture of 1 to 2 x 10^8 cells/ml should be used.

Effect of media on survival and mutation rates. From studies of effects of the absence of glucose, amino acids, and thymine on the treatment of auxotrophs of S. typhimurium KSU#2296, it has been found that mutation and killing can occur in the absence of these nutrients. Therefore, minimal medium, TM buffer, and nutrient broth were tested to select one in which the NG treatment could produce an optimal number of auxotrophic mutants.

In each of these media S. typhimurium LT2 was treated with 20 µg/ml of NG. The results are shown in Table I. There was no significant difference between the type of media used in the number of auxotrophs produced. However,

Figure 4

Survival and reversion effect of NG on S. typhimurium KSU#244 (rua-2) at different phases of growth. Aliquots were taken at 30 minute intervals and treated with 20 µg/ml of NG for 15 minutes.

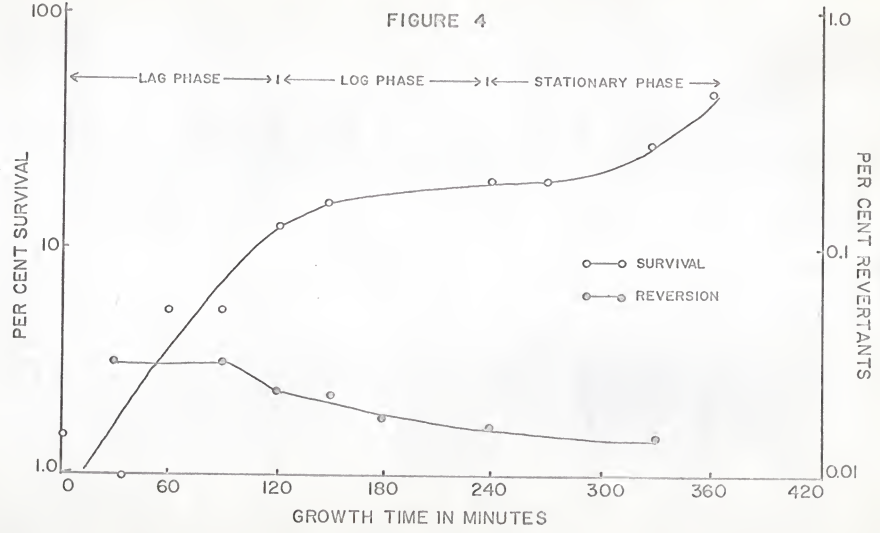


FIGURE 4
LAG PHASE
LOG PHASE
STATIONARY PHASE
PER CENT SURVIVAL
PER CENT REVERTANTS
100
10
1.0
1.0
0.1
0.01
SURVIVAL
REVERSION
0
60
120
180
240
300
360
420
GROWTH TIME IN MINUTES

TABLE I

Survival and mutagenesis as a function of type of medium following a 30-minute treatment with NG.

Medium	NG concentration in μg/ml	Per cent survival*	Per cent auxotrophs among survivors
Minimal	25	17 ± 2	15 ± 2
	10	64 ± 8	12 ± 3
TM buffer	25	10 ± 2	8 ± 2
	10	44 ± 8	9 ± 3
Nutrient broth	25	2.5 ± 0.1	17 ± 4

*All uncertainty intervals are expressed as standard errors, and P = 0.05 unless specified.

the per cent survival varied significantly, with minimal medium giving the highest per cent survival. Adelberg et al. (1965) showed that survival and mutation rate increased greatly when E. coli K12 was treated with TM buffer rather than in minimal medium. This was not observed here in the case of S. typhimurium LT2.

Effect of pH of medium on survival and mutation rates. It has been shown that NG is most stable at pH 5.5 and that it decomposes at higher pH conditions (Mandell and Greenberg, 1960; and our own findings). These experiments showed that upon treatment of cells (S. typhimurium LT2) in minimal medium of pH 5.5, 6.2 and 7.0 there were no significant changes in killing; however, there was a slight but significant increase in per cent auxotrophs obtained among the survivors with the increase in pH of the medium (Table II); therefore, minimal medium of pH 7.0 was used for all NG treatments in order to obtain the highest percentage of auxotrophs. Medium of pH values higher than 7.0 had not been used because precipitation of salts in the minimal medium occurred at pH 7.6.

Effect of time of NG treatment on survival and mutation rates. The S. typhimurium LT2 culture was treated with 25 µg/ml of NG for a period of 60 minutes. Samples were removed and plated at various intervals. The results in Table III show that the per cent auxotrophs among survivors is not significantly different for a period of 45 minutes in the presence of NG, while the per cent survival decreases significantly with the increase of time of treatment. Since there were no significant differences in the per cent auxotrophs produced between 15 to 30 minutes of treatment, a 30-minute treatment was chosen for subsequent experiments.

TABLE II

Survival and mutagenesis as a function of pH of minimal medium following a 30-minute treatment of S. typhimurium LT2 with 25 μg/ml of NG.

pH	Per cent survival	Per cent auxotrophs among survivors
5.5	14 ± 3	9 ± 1
6.2	15 ± 2	11 ± 1
7.0	17 ± 2	15 ± 2

TABLE III

Survival and mutagenesis of S. typhimurium LT2 as a function of time in the presence of 25 µg/ml NG.

Time in minutes	Per cent survival	Per cent auxotrophs among survivors
5	82 ± 8	10 ± 3
15	35 ± 4	11 ± 3
30	11 ± 2	13 ± 3
45	9.6 ± 0.9	15 ± 3
60	6.8 ± 0.7	8 ± 2

In another experiment, S. typhimurium KSU#2l4 (gua-2) was treated with 25 µg/ml of NG for a period of 120 minutes. Samples were taken at various intervals and plated on nutrient agar for survivor counts. The lethal effect of NG decreased after the initial killing (Fig. 5). The possible causes for this leveling effect may be (1) the occurrence of a fraction of resistant cells, (2) a breakdown of the killing agents to a low or non-lethal concentration, or (3) a combination of both.

Effect of NG concentration on survival and mutation rates. S. typhimurium LT2 was treated with different amounts of NG, and the results are shown in Table IV.

The per cent survival of cells showed a significant decrease with increase in NG concentration. However, for the production of auxotrophs from S. typhimurium LT2, there was no significant increase in per cent auxotrophs obtained by increasing the concentration of NG from 10 to 100 µg/ml.

A possible explanation for this discrepancy might be that NG was carried over in samples for plating. To test this, NG treated cells were washed before making dilutions and plating. There were no significant differences in the per cent mutants and per cent survivors obtained from the washed and unwashed cells. Therefore, increased lethality was not a result of NG carried into the plating medium.

From this experiment, a NG concentration of 10 µg/ml was found to be optimal for the auxotroph production from S. typhimurium LT2, since at 5 µg/ml of NG there was a highly significant drop in auxotrophs produced among the survivors, while at 25 µg/ml there was no significant increase in auxotrophs but a definite increase in lethality.

From the above studies it was found that NG (above 1 µg/ml) was both

Figure 5

Survival as a function of time of treatment with NG. *S. typhimurium* KSU#244 (*gua-2*) was treated with 25 µg/ml of NG in minimal medium supplemented with glucose and guanine.

FIGURE 5

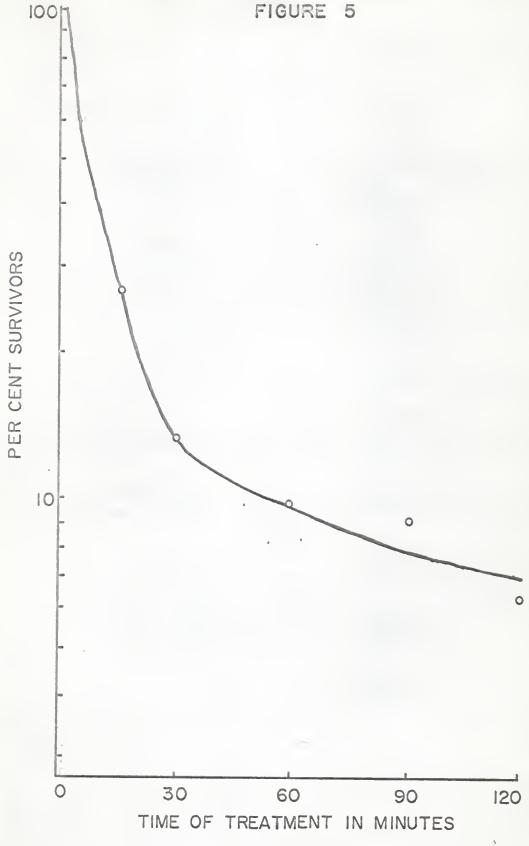

100
10
PER CENT SURVIVORS
0
30
60
90
120
TIME OF TREATMENT IN MINUTES

TABLE IV

Survival and mutagenesis as a function of NG concentration following a 30-minute treatment of S. typhimurium LT2 in minimal medium.

NG concentration in μg/ml	Per cent survival	Per cent auxotrophs among survivors
100	2.3 ± 0.2	16 ± 2
50	5.6 ± 0.5	16 ± 2
25	17 ± 2	15 ± 2
10	64 ± 8	12 ± 3
5	92 ± 10	2 ± 1
1	95 ± 10	<1

lethal and mutagenic for metabolizing or non-metabolizing and dividing or non-dividing cells of S. typhimurium. Optimum yields of auxotrophic mutants of S. typhimurium LT2 may be obtained by treating a logarithmic culture (1×10^8 cells/ml) with 10 µg/ml in a minimal medium of pH 7.0 at 37°C for 30 minutes. Under these conditions, about 11 per cent auxotrophs can be obtained at a 65 per cent survival level.

Effect of a NG analog, N-ethyl-N'-nitro-N-nitrosoguanidine (ENG), on survival and mutagenesis. N-ethyl-substituted instead of N-methyl-substituted nitrosoguanidine was tested for its mutagenic and inhibitory action on S. typhimurium. The culture KSU#244 (gua-2) was spread on a minimal agar plate supplemented with glucose. The plate was then marked into halves; a crystal of NG was spotted on one half and a crystal of ENG on the other half. Prototrophic colonies of revertants of the gua-2 marker were observed only on the side spotted with NG. No reversion of this marker was induced by ENG.

A 0.1 ml aliquot of a saturated ENG solution was spread on a double-enriched minimal agar plate, and then S. typhimurium LT2 was spread over it. After 2 to 3 days' incubation at 37°C, no small colonies among the survivor colonies or significant killing occurred on the plates. Similar plates with NG added gave large zones of inhibition and colonies of various sizes. These results indicate that ENG has little or no ability to inhibit or to induce mutation in S. typhimurium. Therefore, the mere substitution of the ethyl group is sufficient to inactivate the killing and mutagenic activity; the methyl group is likely to be the active component of the NG molecule.

Lethal and Mutagenic Effects of NG on Bacteriophage

A further attempt to elucidate the action of NG on replicating or non-

replicating DNA was made by treating the lysates of bacteriophage P22, T_4D (temperature mutant) and ØX174 with 100 to 400 µg/ml of NG. After incubating the phage lysate in the presence of NG for 20 to 90 minutes, a 1:10 dilution of the lysate was made into ice-cold 0.1 M Tris-buffer of pH 8.5, and the phage were plated on the appropriate hosts to observe in vitro killing and mutagenic effects of NG on the phage. Phage ØX174 was also treated with nitrous acid and plated for host-range mutants which formed plaques on a strain of ØX174 resistant E. coli C. The results shown in Tables V and VI indicate that NG is non-lethal and non-mutagenic on the phage when applied in vitro at concentrations of 100 to 400 µg/ml of NG for periods up to 90 minutes. However, the in vitro nitrous acid treatment produced a number of host-range mutants equal to one hundred times the number of these mutants produced spontaneously.

The effect of NG in vivo on phage was tested by treating host cells (E. coli C) with 100 µg/ml of NG for 5 minutes, and then adding phage ØX174 at a multiplicity of 1. After 15 minutes, the infected culture was washed and resuspended in Hershey's nutrient broth and incubated until the culture became clear. The lysate was then assayed for host-range mutants. A one hundred-fold increase in host-range mutants as compared to spontaneously induced mutants was observed.

These results suggest that NG is inactive on non-replicating DNA but is able to induce mutation when present during DNA replication.

Interaction between NG and DNA

An attempt was made to alkylate DNA with NG by incubating a solution of highly polymerized salmon sperm DNA in 0.01 M sodium phosphate buffer of pH 6.8 with NG (5.0 mg DNA to 100 umoles NG) at 37°C. After 30 minutes of incubation

TABLE V

Test for lethal effect of NG on bacteriophage in vitro.

Phage	Control titer	NG treated phage		
		Titer after treatment	NG concentration	Time of treatment
P22	1.3×10^{10}	1.4×10^{10}	100 µg/ml	90 min.
T4D	1.2×10^{8}	1.3×10^{8}	400 µg/ml	30 min.
ØX174	7.4×10^{10}	7.8×10^{10}	400 µg/ml	20 min.

TABLE VI

Test for mutagenic effect of NG on bacteriophage.

	T4D No. revertants per 10^6 survivors		ØX174 No. host-range mutants per 10^8 survivors		
	Spontaneous	NG induced	Spontaneous	NG induced	Nitrous acid induced
in vitro	48	42	29	28	2600
in vivo	--	--	16	1800	--

the DNA was precipitated with 95% alcohol and hydrolyzed with 1 N HCl. The hydrolysate was chromatographed with a column of Dowex 50WX8 cation exchange resin. The purines were separated from the pyrimidine nucleotides (Fig. 6) by eluting with 2 N HCl. The 260 mμ radiation absorbing fractions were collected and concentrated. When the concentrated fractions were co-chromatographed with authentic samples of guanine and adenine, the ultraviolet absorbing spots of NG treated material had R_f values of 0.21 and 0.34; the R_f values of the authentic samples of guanine and adenine were 0.27 and 0.42, respectively. The ultraviolet absorbing spectra of the two purine fractions in 0.1 N HCl are shown in Fig. 7. They are identical to those of the authentic samples. Molar extinction coefficients were not obtained, because no quantitative determination had been made of the material separated.

The infra-red absorption spectra of both fractions did not show methyl group absorption bands. Other changes of the guanine and adenine structures have not been analyzed. However, this indicates that methylation of the guanine and adenine moieties of DNA did not occur under these experimental conditions; other modification of the purine molecules cannot be excluded.

Figure 6

Separation of the products of hydrolysis of NG treated salmon sperm DNA by a Dowex 50W X 8 cation exchange column. The fractions were eluted with 2 N HCl.

FIGURE 6

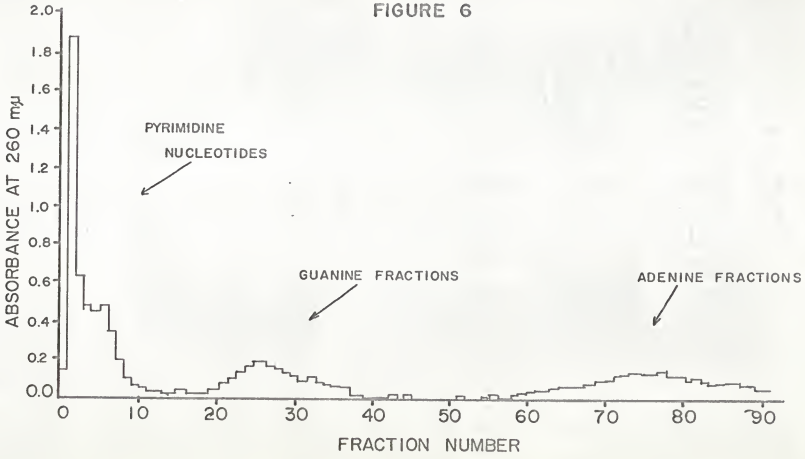
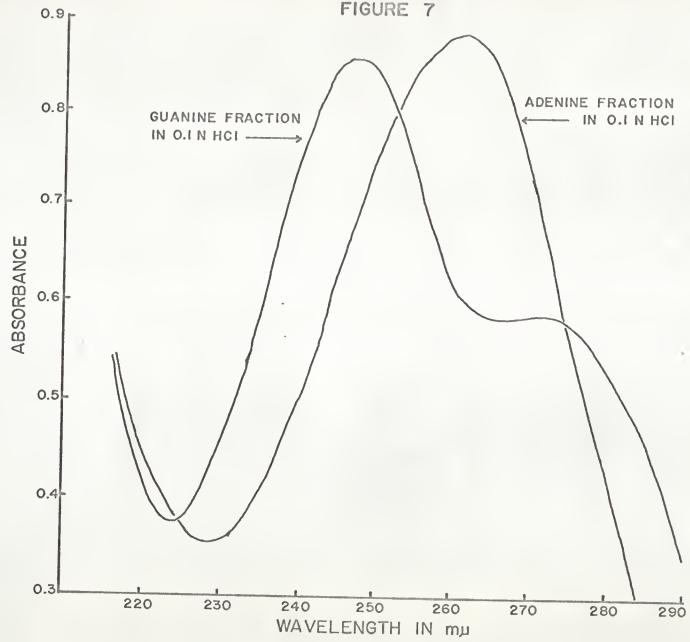

ABSORBANCE AT 260 mμ
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PYRIMIDINE
NUCLEOTIDES
GUANINE FRACTIONS
ADENINE FRACTIONS
0
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

Figure 7

The ultraviolet absorption spectra of the products of hydrolysis from NG treated DNA. The fractions were eluted from the paper chromatogram with 0.1 N HCl. These absorption spectra are identical to those of authentic samples of guanine and adenine, respectively.

FIGURE 7


0.9
0.8
0.7
0.6
0.5
0.4
0.3
ABSORBANCE
GUANINE FRACTION
IN 0.1 N HCl
ADENINE FRACTION
IN 0.1 N HCl
220
230
240
250
260
270
280
290
WAVELENGTH IN mμ

DISCUSSION

N-methyl-N'-nitro-N-nitrosoguanidine has been found to be a powerful mutagen, but its mode of action has not been studied.

The experimental results show that S. typhimurium treated with NG in minimal medium (no energy source) and washed before plating on agar did produce a high percentage of auxotrophic mutants among the survivors. These results indicate that the uptake of NG into the cell may not be an energy requiring process and that DNA replication during treatment is not necessary for NG to induce mutations. Possibly, NG may react with DNA or its precursors and alter them in such a way as to produce template errors or incorporation errors during subsequent replication.

However, NG may also remain bound to the cell and interact with replicating DNA when the chromosome of the survivor eventually replicates to produce progeny. This inability to remove NG completely makes it difficult to exclude the possibility that NG acts solely on replicating DNA. The experiments which involve treating phage with NG seem to support this hypothesis. Bacteriophage treated in vitro with NG did not show inactivation or mutagenic effects. But, when the phage were treated in vivo, host-range mutants, one hundred times those induced spontaneously, were obtained. The explanation for these observations seems to be either that NG is excluded by the protein coat of the phage in an in vitro system or that NG interacts with DNA only when it is replicating inside the host cell. The inhibition of NG by the protein coat of phage so that it will not reach the DNA does not seem to be a favorable hypothesis, because cellular proteins may also inhibit NG, and yet mutations of bacteria and mutation of phage in vivo did occur. Furthermore, if NG interacted with protein molecules to such an extent as to prevent its pene-

tration to the phage DNA, one would expect that inactivation of phage particles might occur owing to the modification of the proteins of the host-phage adsorption apparatus. But no inactivation was observed when phage was treated in vitro with NG. Therefore, the inability of NG to induce mutation when phage was treated in vitro indicates that NG did not interact with resting DNA to induce mutagenesis, and its mutagenic effects on bacteria and on phage treated in vivo suggested that in order to induce mutation an interaction between replicating DNA and NG might be necessary. No information on using NG for the induction of phage mutants has been reported. The in vivo treatment of the phage-host system with NG may be an effective method for obtaining phage mutants.

Mandell and Greenberg (1960) treated cells with NG in a medium of pH 5.5 in which NG is most stable. They then suggested that the NG molecules rather than their breakdown products were responsible for the mutagenesis and toxic effects to the cells. However, it is not hard to conceive that even though the medium was at pH 5.5, inside the cell the pH would be higher (around pH 7), at which NG decomposes slowly into diazomethane which is a methylating mutagen (Kriek and Emmelot, 1963). Also, that diazomethane may be generated through an enzymatic oxidation of NG can be speculated; this mode of action has been reported for dimethylnitrosamine which has been found to be N-demethylated by an enzymatic oxidative process to release a methylating agent, probably diazomethane (Magee and Hultin, 1962; Magee and Farber, 1962). Therefore, the hypothesis that diazomethane is the mutagen instead of the NG molecule itself cannot be excluded. This may also explain why phage treated in vivo gave rise to induced mutants while those treated in vitro did not. The reason would be that an enzyme system inside the cell might be needed to

catalyze the generation of the mutagen diazomethane.

N-ethyl-N'-nitro-N-nitrosoguanidine was found to be inactive in killing and inducing auxotrophic mutants in S. typhimurium. The contrast in activities between the ethyl and methyl analogs is an interesting observation. The use of other analogs may help to elucidate this specificity of action; however, the permeability of the cells to these compounds should also be investigated.

An attempt to show that NG methylates one or more of the bases of DNA failed. DNA treated in vitro with NG was hydrolyzed, the bases and nucleotides were separated, and the bases were examined by their infra-red absorption spectra. No methyl group absorption bands were found in the spectra. However, methylation may be too low to be detected using the above analytical method. Other types of interaction between NG and DNA may have taken place also. Mutational effects often arise from chemical reactions that are below the threshold of detection for any changes in the DNA molecule, especially by the presently available chemical and physical analytical methods.

SUMMARY

From the studies of lethal and mutagenic effects of NG on S. typhimurium the following were observed.

(1) In the absence of glucose, the sole source of external energy, killing of cells induced by NG occurred at a lower rate than when glucose was present. Auxotrophs were produced at roughly the same rate when glucose was either absent or present in the medium.

(2) Similar results were obtained when the amino acid requiring mutant was treated with NG in the absence and presence of the required amino acids.

(3) Thymine starvation also had a lower lethal effect when a thymineless mutant was treated with NG; however, the per cent auxotrophs induced was significantly lowered when thymine was absent in the treatment medium.

(4) Both killing and mutagenesis were more effective in a culture of actively metabolizing and dividing cells than in a culture of stationary phase cells.

(5) Media such as nutrient broth, which support growth best, gave somewhat higher mutation rates but considerably lower survival percentages than minimal medium.

(6) The mutation rate was optimal in a phosphate minimal medium of pH 7.0.

(7) A higher per cent of mutants at a high survival level could be produced by a short treatment time (10 to 30 minutes) with NG.

(8) The optimal NG concentration for the production of auxotrophs was 10 µg/ml.

From these results a set of optimal conditions for the production of auxotrophic mutants of S. typhimurium was established. A yield of 11 per cent auxotrophs among 65 per cent survivors can be obtained by treating logarithmic phase cells in a phosphate minimal medium of pH 7.0 at a NG concentration of

10 μg/ml for 30 minutes at 37°C.

ENG, the ethyl analog of NG, did not inactivate or induce auxotrophic mutation and reversion on S. typhimurium. Therefore, the methyl group is essential both for inducing mutation and for killing.

NG did not inactivate or induce mutation in bacteriophage treated in vitro. However, host-range mutants were produced when phage ØX174 was treated in vivo, indicating that NG was inactive on resting DNA.

The methylation of purine bases was not detected in highly polymerized salmon sperm DNA treated in vitro with NG.

The possible mode of action of NG was discussed.

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MODE OF ACTION OF N-METHYL-N'-NITRO-N-NITROSOGUANIDINE AS A MUTAGEN FOR *SALMONELLA TYPHIMURIUM*

by

SIU WAI SAE

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Department of Bacteriology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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N-methyl-N'-nitro-N-nitrosoguanidine (NG) is a radiomimetic compound which has been found to be a powerful mutagen for Salmonella typhimurium and Escherichia coli. Studies dealing with optimal conditions for producing auxotrophic mutants of S. typhimurium LT2 and the mode of action of NG are reported in this thesis.

By treating S. typhimurium cells in a liquid medium with NG under different sets of conditions, the optimal conditions have been established for obtaining the highest per cent of auxotrophs with only moderate killing of cells. To produce a maximum yield of auxotrophs, a logarithmically growing culture ($1\text{-}2 \times 10^8$ cells/ml) is treated with 10 ug/ml of NG for 30 minutes in a phosphate minimal medium of pH 7.0 at 37°C; a yield of 11 per cent auxotrophs among 64 per cent survivors can be obtained.

Results from the studies of the mode of action of NG are as follows.

(1) Cells treated with NG in a medium deprived of an external energy source (glucose) showed that NG need not be actively taken into the cells to induce lethality and mutation.

(2) Amino acid requiring strains of S. typhimurium were shown to be inactivated and induced to mutate by NG in the absence of the required amino acids when no protein was being synthesized.

(3) Thymineless mutants treated with NG in a minimal medium containing thymine showed a higher killing and higher mutation rate than when treated in a medium without thymine.

These results, therefore, indicate that NG can act on resting DNA to induce mutation, though not as effectively as on replicating DNA.

Results from experiments of treating bacteriophage with NG, however, contradict the above findings. Phages treated in vitro with NG were neither

inactivated nor mutated. However, phages treated with NG *in vivo* (NG added just after infection and removed before lysis of host cells) did produce host-range mutants at a frequency one hundred times that of mutants produced spontaneously. This shows that NG only induces mutation by acting on replicating DNA. A possible explanation of this discrepancy is that NG bound to resting bacterial cells and interacted with DNA on its subsequent replication after being plated on agar.

The ethyl analog of NG has been shown to be inactive both in killing and inducing auxotrophic mutation in *S. typhimurium*, indicating that the methyl group is the active moiety of the NG molecule.

An attempt was made to show the chemical interaction of NG and DNA by simply incubating a mixture of NG and highly-polymerized salmon sperm DNA in solution. The guanine and adenine moieties separated from the hydrolysate of the NG treated DNA were analyzed by their infra-red absorption spectra. No methylation of these bases was found.

These studies show that NG is a powerful mutagen in inducing *S. typhimurium* auxotrophs at a moderately high survival level by acting on replicating DNA. However, the exact chemical interaction of NG and DNA which induces mutation is not known.